Antimalarial and safety evaluation of \textit{Pluchea lanceolata} (DC.) \textit{Oliv.} & Hiern: \textit{In-vitro} and \textit{in-vivo} study

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\textbf{Abstract}

Ethnopharmacological relevance: Many of the effective therapeutic strategies have been derived from ethnopharmacologically used natural products. \textit{Pluchea lanceolata} is an herb employed in Indian folk medicine for malaria like fever but it lacks proper ethnopharmacological intervention.

\textit{Aim of the study:} To evaluate antimalarial and safety profile of \textit{Pluchea lanceolata}: \textit{an in-vitro, in-vivo} for its ethnopharmacological validation.

\textit{Materials and methods:} Methanol, butanol, ethyl acetate, chloroform, hexane extracts and its isolate, \textit{taraxasterol acetate} (\textit{TxAc}) were obtained from air dried aerial part of \textit{Pluchea lanceolata}. These were tested \textit{in-vitro} against chloroquine-sensitive strain of \textit{Plasmodium falciparum} NF54 by measuring the parasite specific lactate dehydrogenase activity. The most potent hexane extract and \textit{TxAc} were further validated for \textit{in-vivo} antimalarial and safety evaluation. \textit{TxAc}, a pentacyclic-triterpene isolated from the most active fraction was further evaluated with special emphasis on inflammatory mediators involved in malaria pathogenesis. Murine malaria was induced by intra-peritoneal injection of \textit{Plasmodium berghei} infected red blood cells to the male Swiss inbred mice. Mice were orally treated following Peters 4-Day suppression test. \textit{In-vivo} antimalarial efficacy was examined by evaluating the parasitaemia, percent survival, mean survival time, blood glucose, haemoglobin and pro-inflammatory immune responses.

\textit{Results:} Hexane extract and \textit{TxAc} showed promising antimalarial activity \textit{in-vitro} and \textit{in-vivo} condition. \textit{TxAc} attributed in inhibition of the pro-inflammatory cytokines as well as afford to significant increase in the blood glucose and haemoglobin level when compared with vehicle treated infected mice. We have not observed the synergistic action of combinations of chloroquine and \textit{TxAc} from our experimental results. \textit{In-vitro} and \textit{in-vivo} safety evaluation study revealed that hexane extract is non toxic at higher concentration.

\textit{Conclusion:} Present study further validates the ancient Indian traditional knowledge and use of \textit{Pluchea lanceolata} as an antimalarial agent. Study confirms the suitability of \textit{Pluchea lanceolata} as a candidate for further studies to obtain a prototype for antimalarial medicine.

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1. Introduction

Malaria is still an important public health problem in many countries of the tropical and subtropical regions of the world with greater mortality in children and pregnant women (Snow et al., 2005). The clinical manifestations of severe malaria are directly correlated with the induction of strong pro-inflammatory immune responses (Schofield and Grau, 2005). Hyperactive immune response is one of the major contributors to cerebral malaria vasculopathy and fatal outcome is generally attributed to the sequestration of activated macrophages, parasitized erythrocytes, and platelets in cerebral vessels (Hunt and Grau, 2003; Haldar et al., 2007). Activation of macrophages is a key event in the pathogenesis of severe malaria in both humans (Baratin et al., 2005) and in experimental models of malaria (Pais and Chatterjee, 2005). \textit{Plasmodium berghei} ANKA infection of C57BL/6 mice, is characterized by the development of strong pro-inflammatory immune responses, including macrophage activation and the production of TNF-\alpha, IL-12, IL-1\beta, IL-6, and nitric oxide (Schofield and Grau, 2005). Anaemia due to the degradation of haemoglobin (Goldberg et al., 1990) is a common feature of severe
malaria and it has been correlated with mortality in many part of the world especially in children and pregnant women (Boeuf et al., 2012). The malaria parasite resides primarily within the host erythrocyte, where it exploits host cell components to meet its needs for life-cycle development. One of the most a predominant and parasite-specific process that occurs during this development is a rapid and organized degradation of the haemoglobin content of infected cells (Rathore, 2007). Hypoglycaemia is also an independent risk factor for death in severe malaria (Achoki et al., 2010; Ogeti et al., 2010). Apart from the malaria parasite, hypoglycemia, degradation of haemoglobin along with pro-inflammatory cytokines are the targets for antimalarial drug discovery.

Pluchea lanceolata (DC.) Oliv. & Hiern. (Family: Asteraceae) popularly known as Rasana is a rapidly spreading perennial rhizomatous weed of sandy or saline soil and distributed throughout the north western part of India, neighbouring Asian countries and also in North Africa (Inderjit and Dakshini, 1998). It is widely used in the treatment of rheumatoid arthritis in Ayurveda, an Indian System of Medicine (Anonymous, 1969). In Ayurveda, the management of malaria considered as ‘Visham jwar’ (Gupta, 2005). Pluchea lanceolata is a one of the ingredient of poly-herbal formulations (Srivastava and Shanker, 2012). Isolation of Taraxasterol acetate (TxAc) from air dried aerial part of Pluchea lanceolata has been depicted in Fig. 1S (Supplementary data).

2. Materials and method

2.1. Collection of plant material

The aerial parts of Pluchea lanceolata were collected from research farm of the Institute. The authenticity of the herb was identified by Dr. SC Singh (Taxonomist) and a specimen was deposited at the herbarium of the Institute voucher number (CIMAP-12555). The aerial part was dried at room temperature for 7 days and ground into powder.

2.2. Extraction and isolation

Air dried and fine powdered aerial part of Pluchea lanceolata (4.5 kg) was extracted with methanol (3 × 7 l) to yield a crude methanol extract (580 g). The methanol extract was concentrated, re-suspended in water and partitioned with hexane, ethyl acetate and butanol. Hexane extract (40 g) was applied to vacuum liquid chromatography (VLC), using silica gel-H and hexane-ethyl acetate as eluent. Elution with hexane, 22–53 fractions has yielded TxAc (350 mg). A compound was identified by comparison of their spectral data with the literature (Akhilasa et al., 1996; Khallilov et al., 2003). The scheme and extraction and VLC separation of isolated compounds i.e., taraxasterol acetate (TxAc) from air dried aerial part of Pluchea lanceolata has been depicted in Fig. 1S (Supplementary data).

2.3. In vitro antiplasmodial activity

Plasmodium falciparum chloroquine-sensitive (NF-54) were cultivated in human B* red blood cells using RPMI-1640 medium supplemented with 25 mM HEPES, 0.2% NaHCO3, 370 μM hypoxanthine, 40 μg/ml gentamicin, 0.25 μg/ml fungizone, and 0.5% albumax at 37 °C (Trager and Jensen, 1976). Culture was maintained in a standard gas mixture consisting of 1% O2, 5% CO2, 94% N2 and culture medium was changed after every 24 h. The culture was routinely monitored through Giemsa staining of thin smears. The culture was synchronized by 5% α-sorbitol treatment to obtain ring-stage parasites (Lambros and Vanderberg, 1979). The test materials were dissolved in dimethyl sulfoxide (DMSO) and further diluted with culture medium to achieve the required concentrations. Parasite growth was determined spectrophotometrically in vehicle control (1% DMSO served as a vehicle control) and drug-treated cultures by measuring the activity of the parasite lactate dehydrogenase (Makler et al., 1993). Briefly, a synchronous ring stage culture with 1.2% parasitemia and 2% hematocrit was incubated in 96-well microtitre plate in different concentrations of test compounds at 37 °C for 72 h. After incubation, plates were subjected to three 20-min freeze-thaw cycles to release the cell content, then cultures were carefully mixed and aliquots of 20 μl were transferred to another microtitre plate containing 100 μl of Malstat reagent (0.125% Triton X-100, 130 mM L-lactic acid, 30 mM Tris buffer and 0.62 μM 3-acetylpyridine adenine dinucleotide; pH 9) and 25 μl of NBT-PE ([3H]methyl blue tetrazolium and 0.24 μM phenazine ethosulphate) solution per well. The plate was incubated in the dark for 30 min and absorbance was recorded at 650 nm using a microplate reader (BMG Labtech Pvt. Ltd, Germany). The absorbance values of test compounds and standards were converted to percentage inhibition using absorbance of control wells. Antiplasmodial activity of the test compounds were calculated as IC50 (Concentration that inhibits 50% growth of parasites) and expressed as mean ± SEM of the three separate experiments performed in triplicate.

2.4. In-vitro cell cytotoxicity

Cytotoxicity was carried out in macrophage cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay as per described by Sharma et al., 2012. Peritoneal macrophage cells (0.5 × 106 cells/well) isolated from mice were suspended in RPMI 1640 medium (Sigma, USA) containing 10% heat-inactivated fetal bovine serum (Gibco, USA) and incubated in a culture 96 well plate at 37 °C in 5% CO2 in an incubator and left overnight to attach. Cells treated with 1% DMSO served as a vehicle control for cell cytotoxicity study. Extracts and TxAc were dissolved in DMSO. Cells were treated (10, 30, 100, 300, 1000 μg/ml) and incubated for 24 h at 37 °C in 5% CO2. After incubation cells with treatment, 20 μl aliquots of MTT solution (5 mg/ml in PBS) were added to each well and left for 4 h. Then, the MTT containing medium was carefully removed and the cells were solubilised in DMSO (100 μl) for 10 min. The culture plate was placed on a micro-plate reader (Spectramax plus 384 with Softmax pro v5.3 software; Molecular Devices, USA) and the absorbance was measured at 550 nm. The amount of color produced is directly proportional to the number of viable cells. Cell cytotoxicity was calculated as the percentage of MTT absorption as follows: Percentage (% of survival) = (mean experimental absorbance/mean control absorbance × 100).

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2.5. Acute toxicity study

Acute oral toxicity tests of the hexane extract was done at on Swiss albino male mice. For this study, mice were administered orally hexane extract (2000 mg/kg) body weight once considered as test group and mice treated with corresponding volume of vehicle (0.7% carboxymethyl cellulose [CMC]; 10 ml/kg body weight) was considered control group. Mice were observed individually, after dosing, at least once during the first 30 min, periodically during the first 24 h and daily thereafter for a total of 7 days (Chanda et al., 2009).

2.5.1. Animals

As experimental hosts, inbred male Swiss albino mice (6–8 weeks old) weighing 18–22 g were procured from institute animal house and acclimatized to the animal room for 5 days prior to experiment. The mice were fed with the pellet mice feed and ad libitum drinking water under standard environmental conditions of 22 ± 3 °C, 12:12 dark-to-light cycle. Animal experiments were carried out as per the approved protocol by the Institutional Animal Ethics Committee (IAEC) followed by the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Government of India (Registration No: 400/01/AB/ CPCSEA).

2.5.2. In vivo antimalarial activity

Plasmodium berghei K-173 strain was used for induction of malaria in experimental mice. The parasites kept in liquid nitrogen were thawed (at 37 °C) and maintained by serial passage of blood from mouse to mouse. The infected blood was collected in heparinizated tubes by the ocular venous sinus and diluted with isotonic saline. The infected blood was collected in heparinizated tubes by the ocular venous sinus and diluted with isotonic saline. The infected blood was collected in heparinizated tubes by the ocular venous sinus and diluted with isotonic saline. The infected blood was collected in heparinizated tubes by the ocular venous sinus and diluted with isotonic saline.

In the in-vivo antimalarial activity was evaluated using the method described by Knight and Peters (1980). In this test, six mice in each group were infected by intraperitoneal injection of 1 × 10⁶ Plasmodium berghei infected erythrocytes, diluted in 0.5 ml of sterile acid citrate dextrose. One hour post infection, hexane extract (10, 30, 100, 200 mg/kg), TxAc (10, 100 mg/kg) alone and in combination with chloroquine (2.5 mg/kg) in two sets of experiments were orally administered once a day for 4 days. On day 4, tail blood was used to make thin blood slides and parasitaemia was counted by microscopic examination of Giemsa stained blood smears. Parasitaemia was determined by counting red blood cells (RBCs) comprising of both the parasitized RBCs and the normal RBCs. Parasitaemia was counted on 4th, 6th, 8th, and onwards up to 28th day. Percentage of parasitaemia was counted based on infected erythrocytes calculated per 100 erythrocytes. Throughout the test, the general condition of the animals in terms of behaviour and clinical signs were also evaluated and the survival of the recovered mice was observed until day 28. Percent suppression was calculated as 100[(A – B)/A], where A is the mean percent parasitaemia of the mice taken as vehicle control and B is the mean parasitaemia in the test group. In addition, mortality in the mice was followed up to 28 days post infection to evaluate the percent survival and mean survival time.

2.6. Blood glucose and haemoglobin level

The possible effect of TxAc on blood glucose, haemoglobin level was assessed on the day 8 of post infection; the day infection was peak in preliminary experimentation. The tail blood was collected for estimation of glucose (Singhal et al., 2011) using glucometer (Ascensia, Bayer) and haemoglobin estimation (Balasubramaniam and Malathi, 1992) using standard drabkins cyanmethemoglobin method as per the manufacturer instruction (Monozyme Ltd, India).

2.7. Quantification of inflammatory mediators using mouse specific ELISA

The second set of experiment was performed to examine the effect of TxAc on inflammatory mediators profile in Plasmodium berghei-induced murine malaria. The mice were infected and treated as per the first set of experiment (procedure mentioned in in-vivo antimalarial study). On day 8 of post infection, the sera from each was isolated from the blood collected from orbital plexus and then animals were sacrificed using cervical dislocation for isolation of whole brain from cranium cavity for quantification of pro-inflammatory cytokines (TNF-α, IFN-γ) using mouse specific ELISA Kit (BD Biosciences, USA) following the manufacturer’s protocol.

2.8. Statistical analysis

Results were expressed as means ± SEM and analyzed using GraphPad Prism 4. Comparisons were made between vehicle control and treatments using by one way analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests. P value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. In-vitro antimalarial activity

*In vitro* antimalarial activity of different extracts and isolate (taraxasterol acetate; TxAc) from Pluchea lanceolata was tested against chloroquine-sensitive Plasmodium falciparum NF54 strain. Methanol, chloroform, butanol, ethyl acetate, hexane extracts and TxAc exhibited IC₅₀ 60.4 ± 0.29, 22.5 ± 0.29, > 100, 7 ± 0.12, 4.9 ± 0.20 and 4.7 ± 10 µg/ml, respectively. Artemisinin and chloroquine, standard anti-malarial drugs exhibited IC₅₀ 0.0067 ± 0.00024 and 0.015 ± 0.00029, respectively. IC₅₀ below 5 µg/ml were considered highly active, IC₅₀ between 5 and 10 µg/ml, moderate activity and IC₅₀ between10 and 50 µg/ml as low activity. The IC₅₀ above 50 mg/ml was considered as not active. Hexane extract and TxAc showed significant antimalarial activity. There are no previous reports on the antimalarial activity of extracts from the aerial part of the Pluchea lanceolata. Hexane extract exhibited highly active antimalarial activity against Plasmodium falciparum NF54 strain may be due to the presence of taraxasterol acetate.

3.2. In-vitro cytotoxicity study

*In vitro* cytotoxicity of hexane extract and TxAc were carried out in the peritoneal macrophage cells isolated from mice using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. The significant change in % dead cell population was not observed (P < 0.05) at any concentration of the treatment when compared with normal cells. Results are summarized in Fig. 1.

3.3. In-vivo acute oral toxicity study

The result of the acute toxicity study showed that a single oral administration of hexane extract (2000 mg/kg) did not produce any mortality, behavioural changes (gait, posture, fur, depression, panting) in the mice as compared to the control group. Similarly, no significant changes were recorded in serum biochemical (total bilirubin, creatinine, triglycerides, SGOT, glucose) as well as haematology parameters (RBCs, WBCs) of the treated group when compared to the control group.
3.4. In-vivo antimalarial activity

Results indicated that the intraperitoneal injection of *Plasmodium berghei* infected RBCs induced the peak parasitaemia on day 8 of post infection. Oral administration of hexane extract (100,300 mg/kg) exhibited significant (P < 0.05) inhibition of parasitaemia on 8 day postinfection as compared to parasitaemia detected in vehicle treated infected control mice, it showed a suppression of parasitaemia of 46.26 ± 1.03 and 71.29 ± 2.01%, respectively. Similarly, TxAc (10,100 mg/kg), chloroquine (2.5, 10 mg/kg) and combination of TxAc (10 mg/kg) plus chloroquine (2.5 mg/kg) also exhibited significant (P < 0.05) inhibition of parasitaemia on 8 day postinfection as compared to parasitaemia detected in vehicle treated infected control mice. The representative data of percentage of parasitaemia and percentage of suppression of parasitaemia are depicted in Table 1 and the effect of TxAc on anti-malarial activity in *Plasmodium berghei*-induced malaria in mice are depicted in Fig. 2A.

The significant improvement in mortality rate was observed in all treated group when compared to vehicle treated infected control. The mean survival time of the mice administered vehicle was 8.66 days whereas, hexane extract (100 and 300 mg/kg) treated group observed 17.33 and 14 days respectively and TxAc (10,100 mg/kg), chloroquine (2.5, 10 mg/kg), and combination of TxAc (10 mg/kg) plus chloroquine (2.5 mg/kg) was 15.50, 16.33,17.66, ≥ 28 and 19.33 days, respectively in Table 1. The result of percent survival of mice treated with TxAc is depicted in Fig. 2B.

3.5. Effect of TxAc on blood glucose and haemoglobin level

The level of glucose and haemoglobin in blood were significantly (P < 0.05) decreased in vehicle treated infected mice when compared with normal mice (Fig. 3). Hypoglycaemia and anaemia is the common feature of severe malaria and it has been correlated with mortality in many part of the world especially in children and pregnant women (Boeuf et al., 2012). The treatments of TxAc, chloroquine and its combination are capable to restore the blood glucose and haemoglobin level towards normal (Fig. 3).

3.6. Effect of TxAc on inflammatory cytokines

Taraxasterol, a pentacyclic-triterpene, was isolated from the Chinese medicinal herb *Taraxacum cinale* has exhibited the anti-inflammatory activity in lipopolysaccharide (LPS)-induced RAW 264.7 murine macrophages (Zhang et al., 2012). These findings suggested that Taraxasterol could be an interesting potential alternative for the treatment of macrophage-mediated inflammatory disorders. Considering these beneficial effects of Taraxasterol, the present study investigated the effect of TxAc on inflammatory mediators in *Plasmodium berghei*-induced murine malaria. Pro-inflammatory cytokine profile in terms of TNF-α and
IFN-γ was estimated from brain homogenate as well as from serum obtained from the malaria infected mice on day 8 when the parasitaemia was in peak. The significant increase in the level pro-inflammatory cytokines (TNF-α and IFN-γ) in brain homogenate as well as in serum was observed in vehicle treated infected control mice when compared with normal mice. Significant ($P < 0.05$) inhibition of TNF-α and IFN-γ production was observed in treatment group when compared with vehicle treated infected control mice (Fig. 4A–D). The plant-derived extracts and compounds are known to exert antimalarial activity by reducing parasitaemia (Kundu et al., 2010; Mota et al., 2012) and modulating the pro-inflammatory cytokines (Agarwal et al., 2011; Mimche et al., 2011; Buapool et al., 2013). Severe malaria is a complex multisystem disorder in which multiple parasite and host factors contribute to disease severity and outcome. Nevertheless, years of studies on malaria have progressively shown an important role for pro-inflammatory cytokines in disease pathogenesis (Hunt and Grau, 2003). High levels of TNF-α and other pro-inflammatory cytokines have long been associated with acute malaria episodes, including cerebral malaria and severe malarial anaemia (Clark et al., 2006; Xu et al., 2013).

4. Conclusion

The results of this study indicated that the oral treatment of hexane extract of Pluchea lanceolata and its isolate, TxAc exerts an antimalarial activity by reducing the parasitaemia, increased mean survival time. TxAc also attributed in inhibition of the pro-inflammatory cytokines as well as afford to significant increase in the blood glucose and haemoglobin level when compared with vehicle treated infected mice. We have not observed the synergistic action of combinations of chloroquine (CQ) and TxAc from our experimental results. In-vitro and in-vivo safety evaluation study revealed that hexane extract and it isolate, TxAc are non toxic at higher concentration. These findings further confirm the ancient
Indian traditional knowledge and use of Pluchea lanceolata as an antimalarial agent. Pluchea lanceolata clearly merits for further investigation towards the management of malaria pathogenesis.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2013.08.003.

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